The interaction of SiO2 nanoparticles with the neuronal cell membrane: activation of ionic channels and calcium influx

The Interaction of SiO_2 Nanoparticles with the Neuronal Plasmamembrane: the Biophysical Basis of Modulation of Ionic Currents and Calcium Influx

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Abstract

SiO_2 nanoparticles (SiO_2 NPs) are one of the most promising tools in the field of nanomedicine. In this perspective the knowledge of the mechanisms of interaction between the SiO^2 NPs and their targets is a prerequisite to the rational design of safe and efficient nanotools for laboratory and clinical applications. We have previously shown that non-toxic doses of 50 nm SiO_2 NPs induce strong and long lasting calcium influx together with membrane potential depolarization and modulation of the electrical activity in GT1-7 neuroendocrine cells. In the present paper we investigated which calcium-permeable channels are responsible for these events. We combined calcium imaging and patch clamp techniques (both whole cell and outside out recordings) with a pharmacological approach to obtain a detailed biophysical characterization of the multiple pathways activated by SiO_2 NPs. We provide evidence that TRPV4, Connexins and Pannexin-like channels are the major components of inward currents elicited by the NPs. Furthermore, pre-incubation with the antioxidant N-acetyl-L-cysteine (NAC) strongly reduced the $[Ca^{2+}]_i$ increase. Our findings suggest that SiO_2 NPs directly activate a complex set of calcium-permeable channels, possibly by catalyzing free radicals production.

Keywords: Neurons, silica nanoparticles, TRPV4, connexin, pannexin, free radicals

Introduction

SiO_2 nanoparticles (NPs) are one of the most promising tools in the field of nanomedicine because of their good biocompatibility[1] and have already been employed in several applications from drug delivery[2] to cell tracking and imaging.[3] We have previously reported[4] that cyanine dye doped, fluorescent silica NPs, displaying high yield and extremely stable photoemission, can be incorporated at nontoxic doses into neuronal cells. These properties are particularly attractive for nanoneuromedicine, that deals with highly sensitive and delicate biological material, both at the cell and at the organ level. In this context, the understanding of the mechanisms of interaction between the NPs and their targets as well as of the cellular and molecular responses elicited by these interactions is a prerequisite to the rational design of safe and efficient nanotools for both laboratory and clinical applications. At the nanoscale, the exploration of the laws that govern the interaction between inorganic particles and biological structures such as membrane proteins is still moving its first steps. A lot of information has to be gathered to set on solid ground the generalized use of nanoparticles in basic and applied biology, and specifically in the highly sensitive field of neuroscience. One of crucial parameters known to be affected by interaction of nanoobjects with cells is the intracellular free calcium concentration, $[Ca^{2+}]_i$, controlling a plethora of signaling pathways, particularly in neuronal cells.[5] We have previously shown that nontoxic doses, 50 nm SiO_2 NPs elicit strong and oscillatory increases in $[Ca^{2+}]_i$ in a neuronal cell line, GT1-7 cells; these signals are reversible even in the continued presence of NPs,[6] fully dependent on calcium influx, and carried at least partially through Transient Receptor Potential V4 (TRPV4) channels even if other, up to now unidentified channels are involved. These data were obtained by means of a calcium imaging approach that, even if useful for a phenomenological description of the process on a population scale, cannot provide a complete and detailed biophysical characterization of the properties of the multiple influx pathways activated by the NPs. The adequate approach is electrophysiology, but data about patch clamp recordings from cells, and particularly neurons, exposed to acute applications of NPs are quite scarce and limited; most of them refer to effects on a specific current, isolated from the whole cellular response.[7] Recently,[8] combining single cell patch clamp measurements with long term recordings of electrical activity with multielectrode arrays we provided evidence that, at nontoxic concentrations,[4] SiO_2 NPs evoke a transient depolarization of the membrane potential, leading to a modulation of action potential firing, without any change in gene expression. In the present paper, we go further and provide evidence that the interaction of NPs with the neuronal membrane directly activates a complex set of ionic channels permeable to calcium; moreover, we describe a main mechanism involved in their activation.

Results

Silica particles

The nanoparticles mainly considered in this work were of bare silica, highly homogeneous in spherical shape and in diameter, of 50 ± 2 nm, of the type already used in previous investigations.[4,6,8] These nanoparticles will be labeled as SiO_2-50 nm NPs. Moreover, selected cell tests were also carried out using two other types of SiO_2 spherical particles: i) nanoparticles functionalized with $–NH_2$ groups, in order to impart different surface properties, namely in terms of ζ-potential, with only a quite limited change in size (55 ± 3 nm)[6] (hereafter: NH_2-SiO_2-55nm NPs), and ii) commercial microparticles about 2 μm in size.[6] TEM and SEM images of the three types of particles are shown in the Supporting Information, hereafter SI, Figure S1.

The material features relevant for this work (ζ-potential, size and dispersion/agglomeration state in the media used for presenting the particles to the cells are listed in Table 1).

SiO_2 NPs elicit long lasting inward currents in GT1-7 cells

In the voltage clamp mode, the holding potential was set at -50 mV (the reversal potential for Cl^- ions with standard Tyrode extracellular and K^+ aspartate intracellular solutions, see Experimental Section), in order to exclude any contribution of Cl^- ions to the NP-induced currents. In these conditions the inward currents could be univocally ascribed to influx of cations into the cell. In 34 cells perfusion with SiO_2-50 nm NPs (20 μg mL^{-1}) induced the activation of an inward current, characterized by a noisy behaviour (Figure 1 A,C); peak current density, expressed as median and interquartile range, was: M = -7.80 pA pF^{-1}, IQR = [-14.80; -4.35] pA pF^{-1} (n = 34). Information about the currents was obtained by subtracting the current-voltage (I-V) relationship recorded in control conditions from the one recorded during the response. The I-V curves of the NP-activated currents of Figure 1 A,C are shown respectively in Figure 1 B,D. In most cases, the I-V curves showed a more or less marked outward rectification; the reversal potential (V_{rev}) was analyzed in 16 cells and was M = -1.7, IQR = [-12.4; +8.7] mV (Table 2).

In 19 out of 24 cells openings of large conductance single channels were clearly detectable (Figure 1C, inset). The amplitude histogram of the elementary events in shown in Figure 1E.

The currents were only partially reversible after NP removal, at least for the duration of the recordings (up to 30 min).

When the cells were challenged with 2 μm SiO_2 microparticles (to assess the role of size), no current was recorded (n= 5, Table 2 and Figure 1F). Similarly, perfusion with NH_2-SiO_2-55nm NPs (to assess the role of surface charge) failed to induce an inward current in 7 out of 12 cells (Figure 1F); in 5 cells, either sporadic events and/or low amplitude inward currents could be observed (Table 2 and SI Figure S2**).**These findings are in agreement with those previously reported[6] with a calcium imaging approach, and provide evidence for a lack of nonspecific perturbation of the cell membrane following exposition to the SiO_2 NPs.

The above protocol was repeated with a lower concentration of the same SiO_2 NPs (0.5 μg mL^{-1}): in 5 out of 29 cells, no detectable change in the current baseline was observed; in the other 24 cells, the NPs elicited currents of similar time course, but with a significantly lower peak current density (Table 2; *p*-value = 0.0004, Mann-Whitney *U* test, two tailed, unpaired). An example is shown in SI Figure S3.

We have previously shown[6] that the calcium signals could be also recorded when the NPs were dispersed in DMEM medium, in which the agglomeration is sharply reduced (Table 1). This finding was confirmed in electrophysiological experiments: when dissolved in DMEM, SiO_2-50 nm NPs (20 μg mL^{-1}) elicited and inward current similar to those observed in Tyrode solution (n = 5). A representative recording is shown in SI Figure S4.

In order to better characterize the inward current, a further set of experiments was performed on cells bathed in an external solution containing 20 mM tetraethylammonium (TEA) and internally perfused with a pipette solution in which K^+ was substituted with Cs^+ (see Experimental Section), to block outward currents through K^+-selective channels. Figure 2 A,C show two typical experiments out of 31 similar recordings. In these conditions the I-V relationships of NP-activated currents were in most cases linear (Figure 2 B); V_{rev} was M= -2.8, IQR= [-10.0; +5.5] mV (n=26). Mean peak current density was significantly lower than in Tyrode solution (Table 2; *p*-value = 0.018, Mann-Whitney *U* test, two tailed, unpaired; since the same control group of peak amplitudes in Tyrode solution has been used twice, *p*-value has been corrected for multiple comparisons). The response was only partially reversible. Single channel opening events could again be resolved (Figure 2 C). The amplitude histogram of the elementary events in shown in Figure 2 D.

Effects of channel blockers

The currents activated by the NPs, both in physiological solutions and in the presence of K^+ channel blockers, showed some peculiar features, such as an irregular evolution in time, a marked noisy

behavior and the occurrence of single channel unitary events of amplitude of about 10 pA in whole cell configuration. These features point to a complex process involving the activation of multiple channel types. In a previous paper[6] we reported that the NPs-induced calcium signal could be reduced by TRPV4 blockers. As for openings of the large conductance channels recorded in whole cell configuration, potential candidates are, among others, members of the connexin (Cx) and/or pannexin (Panx) families.[9] Among connexins, GT1-7 cells express at least Cx26 and Cx43,[10] two proteins that can form gap junctions as well as hemichannels. Panxs are widely expressed in the nervous system,[9c,11] but, to our knowledge, no data are currently available for GT1-7 neurons. Interestingly, in a different experimental model (cultured cortical astrocytes), it has been reported[12] that ultrafine carbon black activates Cx43 and Panx1 channels.

In order to evaluate the involvement of these channels, we used a pharmacological approach. We preliminary analyzed the effects of these blockers on NP-induced calcium signals by means of calcium imaging experiments on cell populations. As a first step we tested the effects of Gd^{3+}, a known blocker of TRPVs and other calcium permeable channels[13] and of several connexins.[14] Its effectiveness as a blocker of pannexins is controversial: some Authors[15] reported lack of blockade of dye uptake through Panx1, while others[16] ascribed a reduction in ATP release from lymphocytes to Gd^{3+} block of Panx1. Since it is known that polyvalent ions can induce nanoparticle agglomeration in suspension[17] we preliminarily checked the aggregation effects by Dynamic Light Scattering at different concentrations of Gd^{3+}. Concentrations in the range 50-100 μM are used by some Authors.[14,16,18] As can be seen in Table 1, agglomeration was strongly increased by 100 μM $Gd3^{+}$, above the detection range of the instrument. At 10 μM, a more detailed analysis revealed a bimodal distribution: while a very small fraction of extremely agglomerated NPs was present, the majority were even less agglomerated than in normal Tyrode (Table 1). For these reasons we employed 10 μM Gd^{3+}, a concentration still compatible with the blocking activity on TRPV and Cx channels.[14b,19] In 4 experiments (354 cells), in the presence of 10 μM Gd^{3+}, NPs failed to induce a detectable increase of $[Ca^{2+}]_i$. Upon blocker removal and in the continued presence of the NPs, we observed a strong increase in $[Ca^{2+}]_i$.(Figure 3A, upper and lower).

A second approach was to combine Ruthenium Red (RR), another blocker of several types of TRP channels including TRPVs[20] and carbenoloxone (CBX), blocker of Cxs and Panx1 channels.[9a, 15,21] Specifically, we used 50 μM CBX and 5 μM RR.[12,22] No relevant change in $[Ca^{2+}]_i$ levels was detected upon addition of the blockers to the medium. On the other hand, the $[Ca^{2+}]_i$ increase induced by SiO_2-50nm NP was strongly reduced but not totally abolished in some cells. For this reason we performed a quantitative analysis by evaluating the area under the curves representing the time courses of the cytosolic calcium concentration after NP administration. Responses induced by

the NPs in the presence of RR and CBX (693 cells from 6 different experiments) were compared with a control group (response in the sole presence of NPs; 457 cells from 6 different experiments). The average $[Ca^{2+}]_i$ increases in the two conditions are compared in Figure 3B, upper; individual traces from one control experiment and one performed in the presence of the blockers are in Figure 3B, lower. For both conditions, areas were evaluated within an integration time interval of 10 minutes starting from the onset of the averaged response and represented by frequency histograms in Figure 3C. Statistical analysis confirmed that the combined administration of 50 μM CBX and 5 μM RR exerted a strong and significant reduction in $[Ca^{2+}]_i$ increase following NP administration (Mann-Whitney *U* Test, *p*-value=$1.4 \cdot 10^{-126}$, effect size r=0.71). Further analytical and statistical details are provided in Supporting Information, Methods.

The same blockers were tested in whole cell patch clamp experiments, with the same intracellular and extracellular solutions as in Figure 2. Figure 3D shows that in the presence of CBX and RR the current was completely and reversibly abolished (n =5). Figure 3 E,F show an example (out of 11 cells) of reversible block of the NP-activated current following the acute application of 10 μM Gd^{3+}.

Biophysical characterization of NPs-activated channels: TRPV4

In order to shed more light on the biophysical properties of the NP activated channels, we performed experiments in the outside-out patch configuration. To confirm that NPs activate TRPV4 channels, we used GSK1016790A, a selective TRPV4 agonist[23] with Cs^+-aspartate solution in the pipette and TEA chloride solution with added MEM supplements in the bath (see Experimental Section). Figure 4A shows the simultaneous activation of TRPV4 channels at V_{hold}= -50 mV in response to 500 nM GSK1016790A. The I-V curve, shown in Figure 4B, is outwardly rectifying with a V_{rev} = -8 mV, as expected for the cationic TRPV4 current.[24] Examples of single channel inward currents and the corresponding amplitude histograms are plotted in Figure 4C (same experiment as in B). Current amplitudes from the traces was respectively -2.2, -1.9 and -1.7 pA, in agreement with previous studies. Figure 4D shows an example of single channel I-V obtained from another outside-out experiment, in the presence of a lower concentration (25 nM) of the agonist. The resulting outward rectifying I-V curve shows a V_{rev} of -4 mV and current amplitudes of -1.8 pA at -50 mV and of +8.1 pA at +100 mV. Moreover, we obtained similar results for both mean patch current and single channel unitary current in experiments in which we applied RR to block the GSK1016790A activated currents (SI Figure S5A, B).

Finally, current amplitudes at -50 mV and +100mV and reversal potential (n=9) were respectively -2.3 ± 0.4 pA, +9.5 ± 0.7 pA and -2.4 ± 4.2 mV. In conclusion, in our experimental conditions, TRPV4 single channel conductance for outward and inward currents values are respectively 90-100 pS and 40-50 pS (Table 3).

Subsequently we challenged outside-out patches with 20 μg mL^{-1} SiO_2 NPs. Figure 4E shows the activation of a cationic inward current at V_{hold} =-50 mV. Importantly, this result obtained on isolated patches provides strong evidence that NPs act via a membrane-delimited mechanism to activate ionic channels. Furthermore, we obtained the I-V relationship and the reversal potential of the average patch NP-activated current (Figure 4F). The resulting curve showed a V_{rev} =4.5 mV; Figure 4G shows the single channel I-V relationship obtained from the same patch (i_{-50} = -1.8 pA, i_{100}= 6.3 pA, V_{rev} = 5 mV). The NP-activated inward current was blocked by RR (Figure 4H). Supporting Information Figure S4C, D show the I-V relationship of respectively the mean patch current and the single channel current blocked by RR.
Finally, amplitude at -50 mV and +100 mV and reversal potential of the single channel currents were respectively of -2.15 ± 0.2 pA, 11.0 ± 1.1 pA and 3.2 ± 2.4 mV (n=11, Table 3). These values do not significantly differ from those obtained for TRPV4 (Welch's *t*-test, *p*-value = 0.67 for comparison at -50 mV, *p*-value = 0.25 for comparison at +100 mV, two tailed, unpaired), suggesting that TRPV4 channels are actually one the calcium-permeable channel types that are activated, via a membrane-delimited mechanism, by SiO_2-50nm NPs in GT1-7 neurons.

Biophysical characterization of SiO_2-NPs-activated channels: large conductance nonselective channels

Whole cell and calcium imaging experiments showed that Gd^{3+} or RR+CBX block almost completely the NP-activated inward currents and calcium influx. These data point to the involment of connexins and pannexins as the main large-conductance calcium-permeable channels activated in response to NPs. In the absence of NPs in the external solution, large conductance nonselective channel activity was detected in 5 out of 42 outside-out experiments. An example is shown in SI Figure S6.
On the other hand, large conductance channel activity in response to NPs perfusion was measured in the majority of the experiments (in 3 out of 4 with 0.5 μg mL^{-1} and in 13 out 14 with 20 μg mL^{-1}). Figure 5A shows an example of the channel activation in the presence of 0.5 μg mL^{-1} NPs. Here and in the trace with an expanded time scale (figure 5B), the activation of unitary single channel events of -12 pA was suddenly followed by a steep activation of additional inward currents of about -67 pA and then of -34 pA. Figure 5C shows the single channel I-V relationship obtained during the

opening of the channel of unitary current amplitude of -12 pA: the conductance was 233 pS and the reversal potential +0.5 mV. Finally, the best fit of the amplitude histogram distribution of the single channel trace shown in the inset (fig 5D; same events as shown at the end of the record of figure 5B) indicates a fully open state of -34 pA (about 680 pS; see below) and two current sub-levels.
In a second set of experiments we analyzed the responses to 20 μg mL^{-1} NPs. Figure 5E shows a series of bursts of single channel currents recorded in the presence of 20 μg mL^{-1} NPs and of the TRPV4 antagonist HC-067047[25] in the bath solution (10 μM), which well illustrates several sub-conductance states and loop-gating events, i.e. transitions between an open and the fully closed state that occur through a number of meta-stable intermediate conductance states with a slow, measurable time course[26]. The best fit of the amplitude histogram distribution (Figure 5F) of the single channel trace indicates a main open state of -15.9 pA, but single channel events of -20 pA and even larger could be detected (arrows in E). Figure 5G, obtained from the same experiment as in E, shows the single channel I-V relationship. The curve was fitted with two regression lines yielding a conductance state of 300 pS (blue line) and another of 220 (red line).
In 5 experiments, NPs activated also channels of higher conductance (see also above, fig 5B) and with different biophysical properties. Figure 6A shows a current trace, recorded in the presence of 20 μg mL^{-1} NPs and 5 μM RR. The I-V relationship in fig 6B shows a strong outward rectification for Vm > 50 mV and V_{rev}= -7.8 mV. In the expanded traces shown in the lower part of fig 6A single channel openings could be observed, with a current amplitude at Vm -50 (red lines) of -29 pA (687 pS) .
In conclusion, the data provide evidence that SiO_2 NPs activate most frequently channels with a conductance in the range of 200-250 pS and in the range of 300-350 pS (Table 3). These data are in agreement with those reported in previous papers for Cx43 and Cx26.[27] In addition, we also recorded nonselective channels with an outwardly rectifying I-V relationship, with a very large conductance in the range of 600-700 pS at -50 mV (Table 3), properties comparable to those described for Panx channels.[11c,26a,28]

SiO_2 NPs induce lipid peroxidation in GT1-7 cells

Production of free radicals, and in particular lipid peroxidation, has been reported to be activated by the interaction of several kinds of NPs with different cell types;[29] this pathway may be in turn involved in the activation of some of the channels we described in the previous sections. To investigate this potential mechanism, GT1-7 were treated with SiO_2-50nm NPs (20 μg mL^{-1}) for 30 min, and the changes in lipid peroxidation were evaluated by means of the Click-iT® Lipid

Peroxidation Imaging kit (see Experimental Section). As shown in Figure 7A, the treatment triggered a significant increase (p-value=0.003) in lipid peroxidation over the basal value, while cell preincubation with 1 mM of the antioxidant N-acetylcysteine (NAC) significantly reduced (p-value=0.04) the response (Welch's F-test and Games-Howell post hoc test).

These protocols were performed by adding the NPs to the medium in the culture dish, differently from all the other experiments in which the NPs were administered by microperfusion. A set of control tests (SI Methods and Figure S7) allowed to confirm that there was no significant difference in the actual NP concentration.

Preincubation with NAC affects the calcium increases induced by 20 μg mL^{-1} SiO_2 NPs

To evaluate the effects of NAC on calcium signals induced by SiO_2 NPs we performed further calcium imaging experiments in which GT1-7 cells were challenged with 20 μg mL^{-1} SiO_2 after 30 min of preincubation with 1 mM NAC. Specifically, we recorded $[Ca^{2+}]_i$ time course from 434 NAC-preincubated cells (from 7 experiments) and compared the responses elicited by NPs with the same set of control traces already used to assess the effect of RR and CBX. NAC preincubation caused a strong reduction of NP-induced calcium signals, as shown by the average traces in Figure 7B; Figure 7C shows 4 representative traces from 2 different experiments (one in control conditions and one with NAC preincubation). The area between each trace and its baseline was evaluated by the same approach previously described; Figure 7D shows the frequency distributions of the areas measured in the two experimental conditions. Similarly to what observed with RR/CBX blockers, the effect of NAC as compared to the control condition was striking, leading to a nearly complete suppression of the response in most of the observed cells (Mann-Whitney U Test, p-value=$9.1 \cdot 10^{-82}$, effect size r=0.64). Further analytical and statistical details are provided in Supporting Information, Methods.

Discussion

The detailed and quantitative assessment of the physiopathological responses elicited by nanoparticles in cells and tissues is the prerequisite for determining which properties (in term of composition, surface, size) have to be selected in view of a safe and effective use of these tools in biomedical applications. The first and critical step of the interaction between these nanoobjects and the cell resides at the plasmamembrane and its rich inventory of proteins that mediate the responses to the changes in the extracellular environment. Among this inventory, a key role is played by ionic

channels, and specifically calcium permeable ones, that can activate both fast responses and long term intracellular signalling mechanisms.[5] In spite of their functional relevance, few reports are available regarding the effects of NPs on neuronal channels: most refer to inhibition of voltage-dependent Na^+ and K^+ channels,[7ab,30] while increases in I_{Na} and I_K by ZnO NPs have been reported at a relatively high concentration (100 μg mL^{-1}).[31] We have previously[6] provided evidence that SiO_2-50nm NPs, at nontoxic doses, activate in the GT1-7 neuroendocrine cell line strong and long lasting increases in $[Ca^{2+}]_i$, at least in part dependent on the activation of the calcium permeable channel TRPV4, an integrator of different physical and chemical stimuli,[32] and only marginally affected by blockers of voltage dependent calcium channels. Therefore the object of the present work was to provide direct evidence, using the patch clamp electrophysiological approach, for the involvement of TRPV4 and to identify the other channels responsible of the NP-activated calcium influx.

In whole cell experiments we have found that SiO_2-50nm NPs (0.5 and 20 μg mL^{-1}) elicit inward currents showing a noisy behaviour, with partial reversibility. The reversal potential, near 0 mV, suggests the involvement of nonselective conductances. The striking observation of single unitary macroscopic events even in the whole cell configuration pointed to the activation of large conductance channels. To dissect the contribution of the different channel types we used a pharmacological approach by means of blockers of TRPV4 channels, and of the connexin and pannexin families of large conductance nonselective channels. By combining calcium imaging on cell populations with whole cell and single channel recordings we show that the three major components of the inward currents elicited by the interaction of the NPs with the neuronal plasma membrane are carried by TRPV4 channels and Cx and Panx-like channels. All three classes of channels described above are known to be involved in a wide spectrum of physiological and pathological processes[11c,25a,30] and characterized by multiple activation mechanisms. In the present case the most relevant is likely related to the production of reactive species, since we have shown that lipid peroxidation is significantly increased after 30 minutes of NP exposure and that preincubation with the antioxidant NAC strongly reduced the NP-induced $[Ca^{2+}]_i$ increase. ROS are involved in the activation of TRPV4;[33] moreover, ROS production has been observed downstream of TRPV4-mediated calcium influx,[34] thus providing a potential amplification mechanism. In addition, some Cxs have been reported to open in response to generation of ROS and other reactive species,[9c,35] and the same holds for Panx channels.[9c,36]

A relevant issue in the interpretation of our data is the finding that their activation has been observed also in excised membrane patches, pointing to membrane-delimited pathways. One explanation is based on the existence of plasmamembrane, constitutively active oxidases that

release oxides/peroxides in the extracellular medium;[37] a recent paper[38] has reported that SiO_2 NPs can generate ˙HO in the presence of H_2O_2. Therefore, channels present in an excised patch can be activated by the reactive species produced either locally or in the surrounding environment, derived from the whole cell population. Interestingly, the Authors reported that in contrast with bare SiO_2 NPs, aminated NPs did not catalyze ROS production: this is in agreement with our observation that aminated NPs failed to induce detectable increases in $[Ca^{2+}]_i$[6] and to activate inward currents in most cells tested (see above). Additionally, more than one mechanism may coexist, in particular for TRPV4: as discussed above, its activation could lead to intracellular ROS production, thus explaining the strong contribution of this pathway to the observed cellular responses. The obvious candidate is mechanotransduction, following membrane stretch due to the interaction with the NP. TRPV4 is a polymodal channel, that can give an integrated response to different chemical and physical stimuli.[39] It is involved in the transduction of cell stretch and mechanical pressure, even if it is still controversial whether it is activated directly by deformation of the membrane or by the ensuing release of some membrane component that in turn acts on the channel.[40] Mechanosensitivity has been reported also for Panx channels.[41]

Conclusions

In the present paper we report the first detailed electrophysiological characterization of the ionic currents activated by the interaction of nanoparticles with the neuronal plasmamembrane and identify the major channel types involved. Combining whole cell and single channel recordings with calcium imaging we show that the response can be ascribed to two major components carried respectively by TRPV4 and large conductance channels and point to the involvement of free radicals production as one of the key actors in this process.

A peculiar feature of the results presented in this paper is that they have been obtained at NP concentrations that we have previously shown to be nontoxic;[6.8,42] a key question is whether the same molecular actors may underlie toxicity at higher doses, given the potential amplification pathways described above. In this light, recently[43] it has been reported that SiO_2 NPs inhibit TRPV4 following its activation with the agonist GSK1016790A in airway epithelial cells; however, these findings were obtained with smaller (10 nm) particles with a ζ-potential of 20 mV; of greater relevance, the concentrations used in native cell (100-3000 $\mu g\ mL^{-1}$) have been shown to be well above the threshold for toxicity.[42] It is quite reasonable that at such high doses the interaction of the NPs with the membrane involves radically different mechanisms. In any case, the information

presented here may be of general value, particularly for cells of the nervous system, since a similar pathway has been reported for Panx1 activation by CB NPs in astrocytes.[12]

Experimental Section

Materials

Lab-made silica nanoparticles, in the bare form (i.e. SiO_2-50nm NP) and functionalized at their surface with amino groups (i.e. NH_2-SiO_2-55nm NP) were prepared following the procedure previously reported, ref 4 and ref. 6, respectively. In brief, in both cases SiO_2 NP were prepared by the reverse microemulsion technique, using tetraethylorthosilicate (TEOS) as source of silica. For the functionalization, (3-Aminopropyl) triethoxysilane (APTS) was added to the microemulsion after the formation of NP. Microparticles were purchased from Corpuscular Inc., www.microspheresnanosphere.com

Characterization of (nano)particles

Size and shape of SiO_2 NP were determined by transmission electron microscopy (Jeol 3010, operated at 300 kV). Samples were prepared by dropping of aqueous suspensions of the NP on TEM grids, supporting a lacey carbon film, and waiting until dryness. Histograms of the size distribution of NPs were obtained by measuring ca. 200 particles, and the mean particle diameter (d_m) was calculated as $d_m = \Sigma d_i n_i / \Sigma n_i$, where n_i was the number of particles of diameter d_i. The results are indicated as $d_m \pm$ standard deviation. Microparticles were imaged by SEM (Zeiss EVO 50), observing an aliquot of the sample fixed on a conventional stub through a carbon conducting biadhesive tape.

Dynamic light scattering (DLS) measurements, providing hydrodynamic radius values, were carried out with a 90Plus Par-ticle Size Analyzer, laser wavelength 660 nm, detection angle 90◦, T = 20 ◦C). Materials were suspended (0.1 mg mL^{-1}) in the relevant aqueous media indicated in Table 1. Measurements were repeated in triplicate. In presence of large agglomerates in the micrometer range, in some cases significant differences among repeated measurements occurred, because of the much more complex scattering behavior[44]. The same samples were used for ζ-potential measurements by electrophoretic light scattering (ELS; Zetasizer Nano-ZS , Malvern Instruments). Results are reported as mean value ± standard deviation of five separate measurements each resulting from 10 runs.

Cell culture

GT1–7 cells, an immortalized line derived from highly differentiated mouse gonadotropin-hormone releasing hormone (GnRH) neurons (generously donated by Prof. P.L. Mellon), were plated either on glass 32 mm cover-slips coated with 100 µg mL^{-1} poly-l-lysine (for calcium imaging experiments) or, for electrophysiological experiments, on uncoated plastic dishes (Falcon, Becton Dickinson) at densities of 10,000 cells cm^{-2}. The cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with10% heat-inactivated fetal bovine serum (FBS; Lonza), gentamycin (50 µg mL^{-1}), and glutamine (2 mM) at 37◦C, in a humidified atmosphere of 5% CO_2 in air and then switched for 1 day to 0.5% FBS supplemented with B27 (Invitrogen), and subsequently for 3-4 days to 0.5% FBS, to improve survival and differentiation.

Electrophysiology

Patch clamp

Conventional whole cell patch clamp recordings were performed at 22-25 °C in the voltage clamp mode. In a first set of recordings, the cells were continuously superfused with a standard physiological Tyrode solution of the following composition (in mM): NaCl, 154; KCl, 4; $CaCl_2$, 2; $MgCl_2$, 1; 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES), 5; glucose, 5.5; NaOH to pH 7.35. Composition of the pipette solution was (in mM): KCl, 15; $CaCl_2$, 3; $MgCl_2$, 3; 4(2hydroxyethyl) -1-piperazine ethane sulfonic acid (HEPES), 5; KAsp, 118; EGTA, 5, Na_2ATP, 5, KOH to pH 7.35. Pipettes had a resistance of 2-5 MΩ. In a second set, in order to abolish or reduce the contribution of currents through K^+ channels, the extracellular solution had the following composition (in mM): NaCl, 134; tetraethylammonium chloride (TEACl), 20; KCl, 4; $CaCl_2$, 2; $MgCl_2$, 1; 4(2hydroxyethyl) 1 piperazine ethane sulfonic acid (HEPES), 5; glucose, 5.5; NaOH to pH 7.35. Composition of the pipette solution was (in mM): CsCl, 20; $CaCl_2$, 0,08; $MgCl_2$, 1; 4(2hydroxyethyl) 1 piperazine ethane sulfonic acid (HEPES), 10; Aspartic Acid, 100; CsOH, 100; Na2ATP, 4; pH 7.35. A few control experiments were performed using DMEM as an extracellular medium.

NPs (sonicated for 20 min prior to the preparation of the suspensions) and other agents were dispersed in the solutions at the required concentration. Solutions were applied by means of a microperfusion system connected to a set of five syringes containing the control and test solutions; the perfusion pipette was located at several tens of microns away from the cell to be recorded, in order to minimize mechanical perturbations.

Correction for junction potential was performed analogically. Data were collected with an Axopatch 200B amplifier (Molecular Probes, USA) using Clampex 10.2 and Axoscope 10.2 software and sampled at 10 kHz and 1 kHz respectively. Current voltage (I-V) relationships were obtained from protocols designed to inactivate the voltage-dependent inward currents expressed in these cells (see SI Figure S8).

In the presence of NPs, whole cell recordings lasted from 5 to 40 minutes, durations comparable to those obtained in control experiments in the absence of NPs.

For single channel outside out experiments, patch pipettes, with a resistance of 5-7 MΩ, were filled with the Cs-aspartate solution used for whole cell experiments. In preliminary experiments with the extracellular solution containing 20 mM TEACl, we observed gigaseal (2-10 GΩ) rupture very soon after NPs perfusion. Therefore, in order to increase the duration of the experiments, based on the rationale that NPs are less agglomerated when they are suspended in DMEM,[6] we added MEM supplements (Sigma) to the above solution. A control test (by means of dynamic light scattering, DLS, see SI Methods and Table 1) showed that agglomeration was lower in this solution than in Tyrode solution, even if greater than previously reported for DMEM.[6] Actually, this condition enhanced the stability of the gigaseals, although the duration of the outside out patch recordings was significantly shorter in the presence of 20 µg mL-1 of NPs (median and interquartile range: MNP = 158 s, IQRNP = [99; 216] s, n = 14) than in the presence of pharmacological agents in similar experiments with the TRPV4 agonist GSK1016790A (MGSK = 346 s, IQRGSK = [260; 687] s, n = 28, p-value = 0.022, Mann-Whitney U test, two tailed, unpaired). Data were filtered at 1 kHz and digitized continuously at a sampling frequency of 1 kHz with PClamp Axoscope software. Steady state voltage clamp and ramp protocols from -120 mV to 100 mV were applied and digitized at 10 or 20 kHz with PClamp Clampex software. Data analysis was performed with OriginPro 9.1 software (OriginLab, USA). To obtain the I-V relationship and the reversal potential of the mean current activated by the agonists, we subtracted, if not otherwise specified, the average of at least ten current traces recorded in control conditions during ramp application (red curves in the figure insets), from the average current recorded in the presence of the agonist (black curves in the figure insets). Single channel I-V was obtained by subtracting the baseline (current ramp during closed or blocked state of the channel, red curves in the insets) from a current ramp recorded during channel opening (black curves in the insets).

Calcium imaging

Cells were loaded with the Fura-2 acetoxymethyl ester (2.5 M, 45 min, 37◦C) and subsequently shifted to a standard physiological Tyrode solution of the following composition (in mM): NaCl, 154; KCl, 4; $CaCl_2$, 2; $MgCl_2$, 1; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 5; glucose, 5.5; and NaOH (pH 7.35). The NPs were sonicated and dispersed in the Tyrode solution at the required concentration. The solutions were applied with a microperfusion system. Cells were imaged every 3 s at 37◦C using a monochromator system attached to an inverted microscope with a 20× objective (SFluor, Nikon). Images were acquired using an enhanced CCD camera (PCO) and the Metafluor software (Universal Imaging Co.).

Assessment of lipid peroxidation

GT1-7 cells were seeded at the initial density of 35000 cm^{-2} in 96 well plates in DMEM supplemented 10% FBS (Lonza). The medium was changed with DMEM plus 0.5% FBS supplemented with B27 (Invitrogen) for 24 hrs and substituted with DMEM 0.5% FBS for three days. Lipid peroxidation was analysed using the Click-iT® Lipid Peroxidation Imaging kit (ThermoFisher). The Click-iT® Lipid Peroxidation Imaging Kit-Alexa Fluor® 488 leverages copper-catalyzed click chemistry and the linoleamide alkyne (LAA) reagent (alkyne-modified linoleic acid) for detection of lipid peroxidation-derived protein modifications in fixed cells. Details are given in Supporting Information Methods.

Statistical data analysis

All samples were first tested for normality (Shapiro-Wilk test) and for homogeneity of variance (Levene's test). Mean and standard error of the mean (SEM), rather than median (or trimmed mean) and interquartile range (IQR), depending on the skewness of data distribution, were used as measures of central tendency and dispersion respectively, as detailed along the text. When possible, statistical significance was assessed by parametric tests, otherwise non-parametric alternatives were used as specified case by case. However, all statistical tests were among unpaired samples, two-tailed and a p-value < 0.05 was considered statistically significant. Single channel current amplitudes are represented as mean ± standard deviation (SD), estimated by fitting the amplitude histograms to a sum of Gaussian functions (OriginPro 9.1).

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Figure legends

Figure 1

SiO_2-50nm NPs activate inward currents in GT1-7 cells. A,C: two examples of whole cell currents recorded following administration of 20 μg mL^{-1} of SiO_2-50nm NPs. Voltage clamp recordings, V_h = -50 mV. Inset in C: a large conductance event observed during a whole cell recording. B,D: I-V relationships obtained from the experiments in A,C. The red curves were obtained subtracting the currents activated by the voltage ramps applied before NP administration (gray) from those recorded during the response (black). E: Amplitude histogram of the large conductance events shown in the inset of C: the resulting amplitude is 17.2 pA. F. Stimulation with either SiO_2 microparticles (diameter 2 μm) or NH_2-SiO_2-55nm NPs failed to induce an inward current; subsequent administration of plain SiO_2-50nm NPs elicited a response.

Figure 2

Inward currents activated by 20 μg mL^{-1} of SiO_2-50nm NPs in the presence of block of K+ selective channels. A,C: two examples of whole cell currents recorded following administration of 20 μg mL^{-1} of SiO_2 NPs. Voltage clamp recordings, V_h = -50 mV. Inset in C: large conductance events observed during a whole cell recording. B: I-V relationships obtained from the experiment in A. The red curve was obtained subtracting the current activated by the voltage ramps applied before NP administration (gray) from the one recorded during the response (black). D: Amplitude histogram of the large conductance events shown in the inset of C: the resulting amplitude is 11 pA.

Figure 3

Effects of channel blockers on the increases in $[Ca^{2+}]_i$ and on the ionic currents activated by SiO_2-50nm NPs (20 μg mL^{-1}). A: Gd^{3+} (10 μM) completely abolished the NP-induced increase in $[Ca^{2+}]_i$. Upper: Average response (105 cells) from one experiment representative of 4 (total 354 cells). Lower: three individual traces from the same experiment. B: the combination of 5 μM RR and 50 μM CBX strongly reduced the NP-induced increase in $[Ca^{2+}]_i$. Upper: the comparison of the average of 457 cells from 6 control experiments in which the NPs were administerd in the absence of the blockers (black) and the average of 693 cells from 6 experiments in which cells were preincubated with the blockers prior to NP administration (gray). Lower: two representative traces

from one control experiment and one with the blockers. C: frequency histograms of the areas between the curve of $[Ca^{2+}]_i$ response to NPs and the reference baseline. Black bars: areas measured in control condition (SiO_2 NPs alone). Transparent gray bars: areas measured during NP administration in the presence of CBX and RR blockers. In most of the tested cells NPs did not induce any calcium signal when administered after CBX and RR preincubation (Mann-Whitney *U* Test, *p*-value=$1.4 \cdot 10^{-126}$; effect size *r*=0.71). D: reversible block of the NP-activated inward current by 5uM RR and 50 uM CBX. E: reversible block of the NP-activated inward current by 10 uM Gd3+. F: I-V relationships of the currents activated by the NPs in the absence (black) and in the presence of Gd+ (red)

Figure 4

SiO_2-50 nm NPs open TRPV4 channels in outside-out patches. A) Currents activated by the TRPV4 specific agonist GSK1016790A (500 nM) at V_{hold}= -50 mV. B) I-V relationship of the patch average current obtained subtracting the red trace to the black trace shown in the inset. C) Examples of TRPV4 single channel currents and the corresponding amplitude histograms. D) Single channel I-V relationship obtained subtracting the red trace (inset, baseline) from the black trace recorded in the presence of GSK1016790A (25 nM). E) Currents activated by 20 µg mL^{-1} SiO_2 NPs at V_{hold}= -50 mV. F) I-V relationship of the patch average current activated by SiO_2 NPs obtained subtracting the red trace from the black trace shown in the inset. G) Single channel I-V relationship obtained subtracting the red trace (baseline) from the black trace shown in the inset. H) Ruthenium red (5 µM) blocked NP-activated TRPV4 channels.

Figure 5

SiO_2-50 nm NPs open large conductance channels in outside-out patches. A) Currents activated by 0.5 µg mL^{-1} SiO_2 NPs at V_{hold}= -50 mV. B) Single channels currents recorded between and during the last 4 voltage ramp of figure A in more expanded scales. Red lines indicate the main current levels. C) single channel I-V relationship obtained by subtracting from the second current ramp (inset, black trace) of B the first one (inset. red trace). The dotted red line represents the regression line obtained by fitting the curve from -50 mV to +40 mV. D) Amplitude histogram, fitted by the sum of 4 gaussian curves, of the single channel trace shown in the inset. E) Single channel currents activated in response to 20 µg mL^{-1} SiO_2 NPs at V_{hold}= -50 mV and (F) the corresponding amplitude histogram. (Arrows indicate single channel events of about -20 pA). G) I-

V relationship of the channel in E), obtained by subtracting the baseline from a current ramp recorded during channel opening (respectively red and black trace in the inset).

Figure 6

SiO_2-50 nm NPs open Panx-like channels in outside-out patches. A) Currents activated by 20 μg mL^{-1} SiO_2 NPs at V_{hold}= -50 mV in the presence of 5 μM RR. Lower traces are single channels currents recorded between and during the voltage ramps in more expanded scales. B) I-V relationship obtained by subtracting the baseline (red trace, inset) from the averaged current obtained from the ramps shown in A (black trace, inset):

Figure 7

SiO_2-50 nm NPs induce lipid peroxidation and this pathway is strongly involved in NP-activated calcium influx.

A) 30 min preincubation of GT1-7 cells with 1 mM NAC prevented the increase in lipid peroxidation induced by 20 μg mL^{-1} SiO_2 NPs (**p*-value=0.04, ***p*-value=0.003, Welch's *F*-test and Games-Howell post hoc test). B) Preincubation with NAC strongly reduced the calcium influx induced by NPs as can be seen from the comparison between the average of 434 cells from 7 experiments with NAC (gray trace) and the average of the same control traces already used as reference for RR and CBX blockers (black trace, 457 traces from 6 experiments). C) Two representative traces from one control experiment compared with two from one experiment with NAC. D) Frequency histograms of the areas between the curve of $[Ca^{2+}]_i$ response to NPs and the reference baseline. Black bars: areas measured in control condition (SiO_2 NPs alone). Transparent gray bars: areas measured during NP administration after NAC preincubation. Significance of NAC effect on calcium signals induced by NPs was tested through a Mann-Whitney *U* Test (*p*-value=$9.1 \cdot 10^{-82}$, effect size *r*=0.64)